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(54) **Cell culture substrate cell sheet, cell cluster and preparations thereof.**

(57) The invention provides cell culture substrates, cell sheets, cell clusters and preparations thereof using temperature-responsive polymeric compounds.

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circulated through the interior of the hollow fiber to supply nutrients and remove waste materials through the hollow fiber membranes, 5) packed glass bead type where cells are in contact and adhered to the packed glass beads and the culture medium is circulated among them, 6) microbead type where microbeads are suspended in the culture medium to attach the cells on the surface of the microbeads which are agitated to culture the cells.

As mentioned above, prior attention has been mainly paid to morphological design of the cell culture device from the view point of effectiveness in nutrient supply and in waste removal. Recently however, it has been found that it is almost impossible to maintain cell viability and functions for a long period only by controlling the efficiency of nutrient supply and waste removal, but the cell culture substrate is a key to control the cell viability and functions for anchorage dependent cells. Therefore, research on the relationship between the property of cell culture substrate and cell functions has been actively carried out.

In the past, polystyrene is most widely used as a material of cell culture substrate because of its optical transparency, non-toxicity, excellent mechanical properties, good moldability and low price. However, the cell adhesion process which leads to the cell proliferation process is significantly inhibited on the surface of the polystyrene culture substrate because of its hydrophobicity. Therefore in order to improve the cell attachment and proliferation, the modified hydrophilic polystyrene which is endowed with negative charges by corona discharge treatment, has been developed and widely used as a cell culture substrate. However, it was found that the above-mentioned modification of polystyrene is still not enough for cells to express and maintain their specific functions.

Recently, the study to bring the cell culture substrate closer to the *in vivo* environment around the cell has started in order to improve cell functions such as attachment, proliferation, differentiation, and production ability of cell products. Namely, the study is to incorporate the substances which effectively control the cell functions into the cell culture substrate. The most typical substance to control the functions is extracellular matrix. Study of the function of extracellular matrix *in vivo* has progressed rapidly in recent years. It has become clear that it plays, not only a simple passive role such as supporting the cells and fixing the cells as known in the past, but also has a function in actively controlling or regulating cell functioning. Although a number of extracellular matrix components have been identified, the most important component is collagen. In addition it has been discovered that there are more than ten different types of collagen each of which is synthesized by a certain definite cell and is located in a certain tissue playing the role of controlling different cell functions. Even with the same type of collagen, modification by introducing a variety of functional groups or modification of higher order structure can cause different effects on the cell functions. As well as collagen, extracellular matrix components such as fibronectin, laminin, thrombospondin, vitronectin, proteoglycan and glycosaminoglycan have been identified. These have specific binding sites relative to the collagen and cell membrane and also play an important role in the cell attachment and proliferation.

Furthermore, except for the above-mentioned extracellular matrices, there are some other substances which effectively control cell functions such as attachment, proliferation and differentiation. They are gelatin which is a thermally degenerated collagen, lectins which bind specifically to sugar moiety on the cell membrane, anchorage oligopeptides which are the binding sites of anchorage proteins such as fibronectin, and adhesive protein isolated from a shellfish.

As examples of the culture substrates combined with these substances which control the cell functions, collagen-coated substrate (K. Yoshizato, et al., *Annals of Plastic Surgery*, **13**, 9, 1984), fibronectin-coated substrate (F. Grinnell, *Expl. Cell Res.* **102**, 51, 1976) and the substrate coated with adhesive protein of a shellfish (P. T. Picciano, et al., *Developmental Biology* **22**, 24, 1986) have been developed, and some improvements in cell attachment and proliferation have been found.

Furthermore, recently the culture substrate coated with polystyrene containing galactose-derivative group as a side chain has been developed and some improvements in the attachment and life of hepatocytes have been recognized (T. Akaike, et al., *Jpn. J. Artif. Organs*, **17**, 227, 1988). By using the cell culture substrates mentioned above, recently it has become possible to culture the cells which have not been able to attach and proliferate on the prior culture substrate such as glass or polystyrene.

However, despite these advancements in culture devices and substrates, the current cell culture technology has the following crucial problems still.

The distinct feature of the culture of anchorage dependent cells is that the cells stop further proliferation if the cells proliferate and completely cover the surface of the substrate. This is called contact inhibition. Therefore, the passage process, that is, the process to detach the cells from the old substrate and then to transfer the detached cells to a new substrate is necessary in order to continue the proliferation. In the past, proteolytic enzymes such as trypsin and collagenase, and EDTA as a calcium chelator were most commonly used for the cell detachment process. However, the prior cell detachment process, like

functions, risk of contamination and laboriousness of operation that accompany cell recovery and passage which are the problems of the cell culture technique of the prior art.

This invention provides: 1) a substrate made from a temperature-responsive polymeric compound having lower LCST than the culture temperature; 2) a carrier formed by coating the said polymeric compound on the supporting material; 3) a carrier made by graft-polymerizing the said polymeric compound on the surface of the supporting material; 4) a carrier which consists of microbeads made from the said polymeric compound having a crosslinked structure; 5) a substrate made from a mixture of the said polymeric compound and substances which effectively control cell functions such as attachment, proliferation and differentiation; 6) a carrier formed by coating the mixture of the said polymeric compound and substances which effectively control cell functions on the supporting material; 7) a carrier formed by laminating a support with the said polymeric compound layer and a layer of substances which effectively control cell functions in sequence; 8) cell sheets and/or cell clusters formed by any of the foregoing.

The temperature-responsive polymeric compound having lower LCST than the culture temperature to be used as substrate in this invention is in a solid state which the cells can utilize as an anchor to adhere and proliferate at cell culture temperature, and will become a soluble state by reducing the temperature below the LCST to permit detachment of cells from the substrate for passage. In addition, in a carrier grafted with the temperature-responsive polymeric compound, the exchange between hydrophilic and hydrophobic states induced by temperature change will detach the cells. In a microbead carrier made from the temperature-responsive polymeric compound with crosslinked structure, the exchange between hydrophilic and hydrophobic states, and between swelling and deswelling states will detach the cells.

Examples of temperature-responsive polymeric compounds that can be used as a substrate in this invention are poly-N-substituted (meth)acrylamide derivatives and their copolymers, polymethylvinyl ether, polyethylenoxide, etherized methylcellulose, and partially acetylated polyvinyl alcohol. Particularly preferred compounds are poly-N-substituted acrylamide derivatives or poly-N-substituted methacrylamide derivatives or their copolymers.

For example, poly-N-isopropylacrylamide (PNIPAAm) is a polymeric compound which shows a negative temperature coefficient of solubility in water (Heskins, M., et al., J. Macromol. Sci.-Chem., A2(8), 1441, 1968). The hydrate (oxonium hydroxide) which depends on the hydrogen bonding formed at a lower temperature between a water molecule and the polymer molecule will decompose at a higher temperature, so that polymers aggregate by dehydration to form a precipitate. Thus, the transition temperature of this hydration and dehydration is called "lower critical solution temperature" or LCST. Thus above the LCST, the said polymer aggregates to form a solid state. But at a temperature lower than the LCST, the polymer dissolves in water.

The present invention takes advantage of such properties of the temperature-responsive polymeric compounds and completes a substrate for cell culture that can attach or detach the cultured cells by merely changing its temperature.

Appropriate temperature-responsive polymeric compounds to be used as substrates of this invention are indicated below, but this invention is not limited to these examples. The LCST of these polymers rise with the sequence of polymers listed below.

Poly-N-acryloyl piperidine, poly-N-n-propyl methacrylamide, poly-N-isopropyl acrylamide, poly-N,N-diethyl acrylamide, poly-N-isopropyl methacrylamide, poly-N-cyclopropyl acrylamide, poly-N-acryloyl pyrrolidine, poly-N,N-ethylmethyl acrylamide, poly-N-cyclopropyl methacrylamide, poly-N-ethyl acrylamide.

The aforesaid polymers may be homopolymers or copolymers with other monomers. Any hydrophilic monomers or hydrophobic monomers can be used as the monomer for copolymerization. Generally speaking, copolymerization with hydrophilic monomer will raise the LCST, and copolymerization with hydrophobic monomer will depress the LCST. With a proper selection of monomers, a copolymer with a desired LCST can be achieved.

Examples of hydrophilic monomers are N-vinylpyrrolidone, vinylpyridine, acrylamide, methacrylamide, N-methyl acrylamide, hydroxyethyl methacrylate, hydroxyethyl acrylate, hydroxymethyl methacrylate, hydroxymethyl acrylate, acrylic acid and methacrylic acid having acidic groups and its salts, vinyl sulfonic acid, styrylsulfonic acid and N,N-dimethylaminoethyl methacrylate, N,N-diethylaminoethyl methacrylate, and N,N-dimethylaminopropyl acrylamide having basic groups and their salts, but it is not limited to these compounds.

Examples of hydrophobic monomers are acrylate derivatives and methacrylate derivatives such as ethyl acrylate, methyl methacrylate and glycidyl methacrylate and so on, N-substituted alkyl (meth) acrylamide derivatives such as N-n-butyl (meth) acrylamide and so on, vinyl chloride, acrylonitrile, styrene, and vinyl acetate and so on, but it is not limited to these compounds.

In general, a copolymer with a monomer having a basic group is desirable because it may enhance

1) sheets or films, such as culture dishes, 2) hollow fiber membranes or flat membrane types, and 3) microbeads, and so on.

Besides, graft polymerization methods using ultraviolet light, x-ray, gamma-ray or electron beam can be used.

5 Crosslinking structures can be formed by using a method of introducing the crosslinking structure during polymerization of the monomer or by using a method of introducing the crosslinking structure after completion of polymerization. Either method can be used in this invention.

Specifically, the former method is carried out by copolymerizing bifunctional monomers. For example, N,N-methylenebisacrylamide, hydroxyethyl dimethacrylate or divinylbenzene can be used. With the latter
10 method, it is common to crosslink the molecules by light, electron beam and gamma-irradiation.

On the other hand, the present invention also takes advantage of the properties of temperature-responsive polymeric compounds and has completed a cell sheet and/or cell cluster. After forming a cell monolayer on the substrate of the temperature-responsive polymeric compound with or without substances such as the extracellular matrix by culturing cells at a higher temperature than the LCST, the cell sheet can
15 be prepared by detaching it from the substrate by lowering the temperature below the LCST. In order to recover a cell sheet formed on the prior substrate, cell detaching agents such as trypsin are necessary. The prior cell detaching agents destroy not only junctions between cells and the substrate but also junctions between individual cells. Accordingly, it was impossible to prepare an excellent self-supporting cell sheet by the prior art. Furthermore, the prior cell detaching agents significantly damaged the cell membrane and
20 membrane-bound receptors.

On the other hand, in this invention it is possible to detach and recover a cell sheet from the substrate by merely changing the temperature instead of using the prior detaching agents. This invention first enables the formation of an excellent self-supported cell sheet which was almost impossible to make by the prior
25 detaching agents. Also, this invention can keep viability and cell functions of the cell sheet due to no use of the prior detaching agents. This invention also enables the formation of an excellent self-supported cell sheet which was almost impossible to make by the prior detaching agents. Furthermore, this invention can significantly simplify the prior complex cell detaching process where the cell washing process and trypsin adding process are necessary. This means that this invention can markedly reduce the possibility of contamination which is a lethal problem in cell culture technology.

30 The cell detached from the substrate by the method in this invention were sheet-like immediately after the detachment, but if the cell sheet was transferred to a non-anchorage hydrophobic dish, the cell sheet gradually rolled up and finally changed to a cell cluster. It is a matter of course to be able to keep sheet-like without changing to a cluster if the circumference of the cell sheet is fixed during the detachment process.

The cell density of the thus formed cluster can be in the order of 10^9 cells/ml. This cell density is about
35 100 times higher than the maximum cell density that can be attained by prior culture (order of 10^7 cells/ml). Thus, although it is a simple calculation, it means that the scale of the equipment for production of cell products can be reduced to about a hundredth.

In the prior art, it was impossible to control the size of the cell cluster and also to produce the cluster in mass, since the prior cell cluster was formed by accidental detachment from the substrate (Koide, N. et al.,
40 Jpn. J. Artif. Organs 17 (1), 179, 1988). In this invention, however, it is easy to control the size of the cluster by changing the surface area of the culture substrate where the cell proliferated and covered before detachment and also to make the cluster in mass. In this invention, the size of the cluster ranges from several microns to several mm. Particularly, this invention is more suitable for production of the larger clusters which were not able to be prepared by the prior art. The larger clusters are effective as the
45 prosthesis for diseased or damaged living tissue or organs.

In addition, when the cell cluster was transferred to a new hydrophilic dish, after the cell cluster was preserved on a non-anchorage hydrophobic dish in a CO₂ incubator for a long period using a common culture medium, the cell cluster started to reattach and repopulate on the hydrophilic dish. This evidence shows that the cell cluster can keep its viability and cell function for a long period. This is because the
50 intercellular junctions and membrane-bound receptors of the cell cluster are kept intact by the detachment method in this invention.

The cell culture substrate of this invention enables a very simple cell recovery by replacing the prior cell detaching agents with mere change in temperature and reduce the complexity and risk of contamination of the prior cell culture technology. In addition, the cell sheet and/or cell cluster of this invention showed
55 high cell density, high cellular functioning and excellent self-supporting properties. This means that the cell sheet and/or cell cluster of this invention are strongly available for 1) bioreactors for production of cell products, 2) prosthesis for diseased or damaged living tissues or organs, and 3) simulators to evaluate toxicity and activity of drugs and so on.

of n-BMA, the LCST of the copolymer significantly decreased.

Table II

LCST of Cop(NIPAAm/BMA) in PBS and calf serum		
	LCST (°C)	
Polymer	PBS	Calf Serum
PNIPAAm	28.9 ± 0.1	28.2 ± 0.1
Cop.(NIPAAm/BMA)-1	19.0 ± 0.1	20.5 ± 0.4
Cop.(NIPAAm/BMA)-2	7.8 ± 0.3	13.8 ± 0.2

Example 3

By dissolving PNIPAAm and NIPAAm monomer which were synthesized and used in Example 1, in DMEM containing 10% FCS, 1.0% PNIPAAm and 1.0% NIPAAm, solutions were prepared for cytotoxicity tests. For control, DMEM containing 10% FCS was used. Then, human dermal fibroblasts were dispersed in PNIPAAm, NIPAAm and control solutions so as to form a cell density of about $1 \times 10^5/\text{ml}$ and each solution of 2 ml was poured into the plastic 35 mm cell culture dishes (Falcon Co.). The cells were cultured at 25°C in a CO₂ incubator (air/5% CO₂). After culturing for one or three days, attachment and proliferation of the cells were examined by a phase contrast microscope. The degree of attachment and proliferation were used as a measure of cytotoxicity. The results are shown in Table III. As shown in Table III, no cytotoxicity of PNIPAAm was observed, although strong cytotoxicity was recognized in the NIPAAm monomer.

Table III

Cell attachment and proliferation		
	Cell attachment and Proliferation	
Substance	1-day culture	3-day culture
Control	○	⊙
PNIPAAm	○	⊙
NIPAAm	x	x
⊙ : Excellent ○ : Good x : Poor		

Example 4

The cytotoxicity of Cop.(NIPAAm/BMA)-1 which was synthesized in Example 2 was evaluated by the same method as Example 3 and shown in Table IV. Here, the cell culture was carried out at 17°C so that the polymer can dissolve in the culture medium. As shown in Table IV, no cytotoxicity was observed in Cop.-

above-mentioned process was performed aseptically. Thus, dishes which were coated with a mixture of collagen and PNIPAAm in equal volume in different thicknesses were prepared. Here, the coating thickness was controlled by the volume of the mixture solution poured into the dish. Then, the human dermal fibroblasts which were used in Example 5 were dispersed in DMEM containing 10% FCS to form a cell density of about $2 \times 10^5/\text{ml}$. Two milliliters of the cell suspension kept at 37°C was poured into the dishes coated with the mixture of PNIPAAm and collagen in different thicknesses which were kept at 37°C . The cells were cultured at 37°C in a CO_2 incubator (air/5% CO_2) for 5 days. The relationship between cell proliferation and the thickness of the coating layer is shown in Table V. On the other hand, the relationship between the thickness of the coating layer and the cell detachment which was observed by a phase contrast microscope when the dishes were transferred from 37°C to room temperature, is also shown in Table V. As shown in Table V, the cell proliferation was excellent independent of the thickness and the cell detachment was improved with the thickness reaching a maximum at a thickness of more than $0.7\text{ }\mu\text{m}$.

For a comparative example, using a non-coated 35 mm plastic dish (Falcon Co.), the human dermal fibroblasts were cultured in the above-mentioned manner. After the cells fully covered the dish, the dish temperature was cooled from 37°C to room temperature, but no detachment of cells was observed. Therefore, the following prior cell detachment process was carried out. The culture medium was discarded from the dish and 2ml of PBS was poured into the dish to wash the surface of the cells for removal of trypsin inhibitor contained in the medium and then the PBS was discarded. Then 2 ml of trypsin/EDTA solution (0.05% trypsin, 0.5 mM EDTA) was added to wash the surface of the cells, the trypsin/EDTA solution was discarded. Again 2 ml of fresh trypsin/EDTA solution was added and the solution except 0.5 ml was discarded. Then the dish was incubated at 37°C for 10 minutes. Using a microscope, complete detachment of cells from the dish was confirmed but the detached cells were isolated from each other and did not make a cell sheet. Also, the prior detachment process using detaching agents consists of a lot of procedures and was significantly complex compared to the current process of merely changing the temperature.

Table V.

Dependency of cell proliferation and cell detachment upon the thickness of coating layer of mixture of PNIPAAm and collagen (1/1)		
Thickness (μm)	Cell Proliferation	Cell Detachment
0	⊙	x
0.2	⊙	○
0.5	⊙	○
0.7	⊙	⊙
0.9	⊙	⊙
⊙ : Excellent ○ : Good x : Poor		

Example 8

The aqueous solutions of the different mixtures of PNIPAAm and collagen which were used in Example 7 were prepared. The composition ratio of the collagen to PNIPAAm are shown in Table VI. The dishes coated with these aqueous solutions in a thickness of about $0.9\text{ }\mu\text{m}$ were prepared by the method similar to Example 7. The human dermal fibroblasts were cultured in a similar manner to Example 7. The relationship of the composition ratio of collagen to PNIPAAm with cell proliferation and cell detachment was measured and is shown in Table VI. The cell proliferation rose with the content of collagen and cell detachment

An aqueous solution (0.5%) of atactic polymethylvinyl ether (Tokyo Kasei K.K.) was prepared. The solution was sterilized by autoclaving (121 °C, 20 minutes) and then cooled to redissolved the polymer. The LCST of the atactic polymethylvinyl ether was about 35 °C in PBS as measured by turbidimetry. This solution was mixed with equal volume of 0.5% collagen solution used in Example 7 to prepare a solution containing 0.25% polymethylvinyl ether and 0.25% collagen as the final concentration. By a method similar to Example 7, the dish coated with the mixture to a thickness of about 1 µm was prepared. Then the human dermal fibroblasts used in Example 3 were dispersed in DMEM to form a cell density of about 2×10^5 /ml. Two milliliters of the cell suspension kept at 40 °C was poured into the aforesaid dish kept at 40 °C and the cells were cultured at 40 °C in a CO₂ incubator (air/5% CO₂) for 5 days. The cells covered the dish and the dish was withdrawn from the 40 °C incubator and left at an ambient temperature. The cells were spontaneously detached from the dish and the cell sheet was suspended in the medium.

Example 12

An aqueous solution (0.5%) of PNIPAAm which was synthesized in Example 1 was prepared, and sterilized by filtration through a 0.45 micron filter. Then this solution was mixed with an equal volume of 0.05% gelatin aqueous solution (Iwaki Glass K.K.) and 400 µl of the aforesaid mixture solution was poured into a plastic 35 mm cell culture dish (Falcon Co.) and dried in a clean hood at an ambient temperature. The above-mentioned procedure was carried out aseptically. Using the dish coated with the mixture, of gelatin and PNIPAAm, the human dermal fibroblasts were cultured by the same method as Example 7. After the cell proliferation, the outside of the dish was cooled to about 10 °C and the cell sheet was able to be detached from the dish and recovered.

Example 13

An aqueous 5% solution of PNIPAAm used in Example 1 was prepared and sterilized by filtration through 0.45 micron filter. Then this solution was mixed with aqueous solutions of fibronectin isolated from calf plasma (Nitta Gelatin K.K.) so as to form mixture solutions with different compositions. The plastic 35 mm dishes (Falcon Co.) were coated with these mixture solutions with different compositions and air-dried at room temperature in a clean hood. These procedures were carried out aseptically. By the same method as Example 8, the human dermal fibroblasts were cultured on the dishes with different compositions and the relationship of the composition of fibronectin and PNIPAAm with cell proliferation and cell detachment was studied (Table VII). As shown in Table VII, cell proliferation improved with the composition ratio of fibronectin to PNIPAAm, but the detachment was poorer than collagen.

Table VII

Dependency of cell proliferation and detachment upon composition ratio of fibronectin to PNIPAAm (thickness: 0.9 µm)		
Composition Ratio of Fibrinogen to PNIPAAm	Cell Proliferation	Cell Detachment
0.01/1.0	Δ	Δ
0.04/1.0	○	Δ
0.08/1.0	⊙	x
⊙ : Excellent ○ : Good Δ : Not Good x : Poor		

Table IX

Dependency of cell proliferation and detachment upon composition ratio of concanavalin A to PNIPAAm (thickness: 0.9 μ m)		
Composition Ratio of Concanavalin A to PNIPAAm	Cell Proliferation	Cell Detachment
0.01/1.0	Δ^*	Δ
0.04/1.0	Δ^*	Δ
0.08/1.0	Δ^*	Δ
0.5 /1.0	\bigcirc^*	Δ

*: Although cell attachment was good, no cell proliferation was really observed.

\odot : Excellent

\bigcirc : Good

Δ : Not Good

\times : Poor

Example 16

First, using 0.5% aqueous solution of PNIPAAm prepared in Example 1, the plastic 35 mm dish was coated with PNIPAAm to a thickness of about 0.9 μ m and then 400 μ l of aqueous solution of fibronectin (concentration 0.1 mg/ml) kept at 37°C and used in Example 13 was poured into the aforesaid PNIPAAm-coated dish kept at 37°C and the dish was air-dried aseptically in a 37°C incubator. By this method, the dish having a laminated coating composed of a PNIPAAm layer and subsequently of a fibronectin layer whose fibronectin density is about 5 μ g/cm², was prepared. Using this coated dish, by the same method as Example 7, the cell proliferation and cell detachment profiles were evaluated. As a result, the cell proliferation and simultaneously the cell detachment were excellent.

Example 17

A commercial available polyethylene terephthalate (PET) film (size: 5 cm x 5 cm, thickness: 100 μ m, Toray Ind. Inc.) was inserted into a chamber of plasma irradiation apparatus with an internal electrode (Samco International K.K.). After the interior of the chamber was evacuated to 0.8 Torr, argon gas was introduced into the chamber at a flow rate of 30ml/min and the film was irradiated with plasma (output = 100 Watts, frequency = 13.56 MHz) for 15 seconds. Immediately thereafter, 5% aqueous solution of NIPAAm monomer was introduced to the chamber so as to soak the film in the solution and the polymerization was carried out at room temperature for 16 hours. In advance, from the monomer solution, contaminated air was completely removed by bubbling with argon. After washing the treated film thoroughly with water, it was dried under vacuum at an ambient temperature.

For controls, without introducing NIPAAm monomer into the chamber, the PET film was treated by plasma irradiation in similar conditions. Also, the PET film merely coated with PNIPAAm was prepared by a solvent casting method using PNIPAAm aqueous solution. The contact angles of the above-mentioned films against water 10°C and 40°C were measured and listed in Table X. As shown in Table X, in untreated film and plasma-treated film, no change in contact angle was observed between at 10°C and at 40°C, but in PNIPAAm coated film and PNIPAAm plasma-grafted film, significant differences in contact angle were found. Both films showed hydrophilicity at 10°C and hydrophobicity at 40°C.

On the other hand, the obtained spheroidal fibroblast cluster was cultured on the non-anchorage dish in a CO₂ incubator (air/5% CO₂) for an extra 20 days and then the cell cluster was transferred to the plastic hydrophilic dish (Falcon Co.) and was cultured in similar conditions to the above-mentioned. After a 2-day culture, the cell cluster reattached to the hydrophilic dish surface and repopulated after a 10-day culture.

5 This evidence demonstrates that the cell cluster has proliferation activity even after a long preservation.

For controls, using the dish coated only with collagen prepared in Example 8, in a similar manner to the above-mentioned conditions, the human dermal fibroblasts were cultured. In order to detach the cell sheet which fully covered the dish after a 4-day culture, the prior cell detachment procedures were carried out. The old culture medium was discarded from the dish and 2 ml of PBS was poured into the dish to wash the surface of the cells for removal of trypsin inhibitors contained in the medium and then the PBS was discarded. Then 2 ml of trypsin/EDTA solution (0.05% trypsin, 0.53 mM EDTA) was added to wash the surface of the cells, and the trypsin/EDTA solution was discarded. Again 2 ml of fresh trypsin/EDTA solution was added and the solution, except 0.5 ml, was discarded. Then the dish was incubated at 37°C for 10 minutes. Using a microscope, complete detachment of the cells from the dish was confirmed, but the detached cells were isolated from each other and neither made a cell sheet nor a cell cluster. Accordingly, 15 by this cell detachment method, it was impossible to prepare these cell sheets and cell clusters.

Example 20

20

The fibroblast sheet which was obtained by the detachment procedure in Example 16, was transferred to a non-anchorage hydrophobic dish and cultured in a similar manner to Example 19. After a 2-day culture the cell sheet completely changed to a cluster whose size was about 1 mm. After the cell cluster was preserved on the hydrophobic dish at 37°C in a CO₂ incubator (air/5% CO₂ for 3 months, the cluster was again transferred to a new hydrophilic dish and was cultured. After 2 days, the cluster attached to the dish and started to repopulate. This evidence shows that the cell cluster continues to live even after 3 months of preservation.

30

Claims

1. A cell culture substrate comprising a temperature-responsive polymeric compound having a lower LCST than the culture temperature.
- 35 2. The cell culture substrate of Claim 1, wherein the said polymeric compound is selected from the group comprising poly-N-substituted acrylamide derivatives, poly-N-substituted methacrylamide derivatives, their copolymers, polyvinylmethyl ether and partially acetylated polyvinylalcohol.
3. The cell culture substrate of Claim 1, further comprising substances which effectively control cell functions.
- 40 4. The cell culture substrate of Claim 3 wherein said substance which effectively controls cell function is selected from the group comprising extracellular matrix, gelatin, lectin, anchorage oligopeptide and adhesive protein isolated from shellfish.
5. The cell culture substrate of Claim 4 wherein the said extracellular matrix is selected from the group consisting of collagen, fibronectin, vitronectin, laminin, proteoglycan, glycosaminoglycan and thrombospondin.
- 45 6. The cell culture substrate of Claim 1 wherein the said polymeric compound is coated on the surface of the supporting material.
7. The cell culture substrate of Claim 1 wherein the said polymeric compound is graft-copolymerized on the surface of the supporting material.
- 50 8. The cell culture substrate of Claim 1 wherein the said polymeric compound has a crosslinked structure.
9. The cell culture substrate of Claim 1 wherein the shape at the cell culture temperature is a film, sheet, particle, fiber, flake, sponge or microbead.
10. The cell culture substrate of Claim 3 wherein said polymeric compound and said substance which effectively controls cell function are coated on the surface of the supporting material as a homogeneous mixture.
- 55 11. The cell culture substrate of Claim 3, wherein said polymeric compound and said substance which effectively controls cell function are coated on the surface of the supporting material in a sequential manner.



DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
Y	PATENT ABSTRACTS OF JAPAN, vol. 8, no. 205 (C-243)[1642], 19th September 1984; & JP-A-59 95 930 (KOGYO GIJUTSUIN (JAPAN)) 02-06-1984 * The entire abstract *	1-20	C 12 N 5/00
Y	WO-A-8 808 448 (M. BAY) * The claims; page 2, line 30 - page 3, line 15; page 5, lines 1-17; page 7, lines 30-33; page 9, lines 5-11; page 10, lines 10-29; page 11, lines 3-5; page 12, lines 18-26 *	1-20	
			TECHNICAL FIELDS SEARCHED (Int. Cl.5)
			C 12 N
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 26-06-1990	Examiner RYCKEBOSCH A.O.A.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ----- & : member of the same patent family, corresponding document	